欠損を伴う歯周病患者に対する新たな細胞治療法を開発することである。そのため、自家、及び他家移植モデル作製のための顎骨由来間葉系細胞株を作製すると共に、より安全性の高い生体外ヒト細胞増幅・不死化技術の確立を目的としている。

B. 研究方法（倫理面の配慮含む）

初年度は実績のあるHPV16のE6やE7などのウイルス遺伝子を用い不死化を試みた。次いで、E7+hTERTやBmi-1+hTERTなどによる不死化を試みた。最終年度は、p16と結合できない変異型CDK4とCyclin D1の発現による不死化を試みた。また細胞種特異的なプロモーター化に不死化遺伝子を調節し特定の細胞種のみを不死化できる系の作成を試みた。不死化細胞から不死化に用いた外来遺伝子を除去あるいは発現調節できるよう、Cre-loxPシステムやtetON/OFFシステムを導入し、不死化遺伝子の除去や発現調節による分化能の差を評価できるようにした。Cre-loxPシステムやtetON/OFFシステムを初代培養細胞に簡便に導入できるよう両システムをそれぞれレトロウイルスあるいはレンチウイルスベクターにより細胞集団に効率よく導入し遺伝子発現調節できる系を樹立した。不死化後の細胞の分化能や骨形成能などを齋藤らが樹立した方法により解析した。

（倫理面への配慮）

ヒト細胞全般の不死化研究については各施設の倫理委員会ならびに国立がんセンター倫理審査委員会の承認（承認番号14-69）を得ている。

C. 研究結果

当初は細胞不死化機構解析の進んでいる乳腺上皮細胞などのモデル細胞を用いて多くの不死化遺伝子を種々の組み合わせで導入し評価した。HPV16のE6E7+hTERTは多くの細胞の不死化ができ実績もあるが、E6はp53を不活化するため染色体異常が誘導されやすい。マウスの細胞ではp53の不活化が必要なため主にE6による不活化を進めたが、ヒト顎骨由来骨芽細胞(HAOB3)やイヌ顎骨間葉系細胞などにはE7+hTERT, Bmi1-hTERTなどを試み成功した。HAOB細胞を間葉系幹細胞用培地(MF培地)で培養すると、遺伝子導入なしでも70 PDほど培養可能であるが骨形成能は30 PD程度で失われる。しかし、E7+hTERTを導入したHAOB細胞集団は30PDを過ぎても骨分化能を維持していた。HAOB細胞を長期培養すると脱分化する可能性と骨分化能を有する細胞は30PDほどで老化しその後は骨分化能を持たない細胞のみが増殖する可能性が示唆された。骨分化能を有する細胞のみを不死化するため骨芽細胞特異的なプロモーターとしてオステオカルチンなどのプロモーター下にE7を発現させるベクターを用いて不死化を試みたが、発現レベルが低すぎて成功しなかった。また、E7が分化に悪影響を与える可能性を考え細胞不死化後にCre-loxPシステムによりE7を除去できる系をじゅりつした。しかし、E7除去により急激に細胞増殖停止が起きるため分化能を評価できなかった。一方、より生理的な不死化を目指し、pRbを本来不活化するキナーゼである変異CDK4+Cyclin D1を導入しhTERTとの組み合わせにより不死化する方法にたどり着いた。この遺伝子の組み合わせによりHAOB3を高率に不死化できることが分かった。次に、外来遺伝子の発現を調節可能にするため、Clontech社の最新のtetON/OFF advanceシステムをレンチウイルスベクター化し、初代培養細胞集団をtetON細胞あるいはtetOFF細胞化できるように改良した。tetOFF(tTA)とtetracyclin resposive element (TRE)により発現調節される変異CDK4とCyclin D1を同時に導入しさらに恒常的に発現するhTERTを導入することで不死化細胞を得た。不死化に至る過程に増殖遅延は一切見られず、初代培養HAOB3細胞が集団として不死化されたことが示唆された。

これらの細胞では変異CDK4とCyclin D1が高発現しておりp53, p16なども発現が高くなっていることが示された。この細胞にドキシサイクリン（DOX）を高濃度に添加すると、変異CDK4ならびにCyclin D1の発現が低下し増殖も遅くなりやがて増殖停止した。

D. 考察

種々の不死化法を試みたがHAOB3などのヒト細胞にはHPV16のE6E7やBmi-1などを用いた方法よりも変異CDK4 +Cyclin D1+hTERTによる不死化が最も有効であることが示唆された。これは、不死化に必要

なp16/pRb経路の不活化に本来のpRb不活化キナーゼであるCDK4 +Cyclin D1の組み合わせを用いたためでではないかと推測している。ヒト卵巣表層上皮細胞をこの遺伝子の組み合わせで不死化すると正常２倍体を長期間維持できることを明らかにしている。これらの細胞ではp53発現が増加しており、正常なp53経路が染色体の安定性に極めて重要であることを示唆している。HAOB細胞はhTERT+変異CDK4で延命できなかったが、cyclin Dを追加することで効率に延命することが明らかになった。さらにhTERTの追加導入により不死化に成功した。H19年度にCre-loxPシステムによる外来遺伝子の除去を試みたが除去後は急激に増殖停止するため分化誘導能を調べることも困難であった。そこでH20年度にはtetON/OFFシステムを導入することで外来遺伝子レベルをDOX濃度により自由に調節できる系を樹立し問題点を解決することができた。今後、目的に即した不死化遺伝子の至適発現レベルを明らかにしていきたい。

現在、不死化細胞の移植やin vitro分化誘導系における骨形成能や外来遺伝子発現による分化・骨形成能への影響の解析を齋藤が進めているが本細胞の有用性が示されてきている。

E. 結論

種々のヒト正常細胞を用い、種々の遺伝子の組み合わせによる細胞不死化を進めてきた。本研究に用いたHAOB細胞などのヒト細胞では変異CDK4+Cyclin D1+TERTの組み合わせで高率に不死化でき、不死化細胞ではp53とp16の高発現が維持されているもののアポトーシスなどは誘導されなかった。本法は正常p53の高発現により不死化細胞は正常２倍体を維持している可能性が高く、染色体異常も起きにくいことが推測される。これまでの細胞不死化法では脱分化を伴うことが多かったが、tetON/OFFシステムの導入により、不死化遺伝子の発現量をDOX添加により調整できることから不死化維持に最低限必要な遺伝子発現レベルを調節することも可能である。また、不死化遺伝子が分化に悪影響を与えたりする可能性があるが、これも分化誘導時に不死化遺伝子発現レベルを低下したり、なくしたりすることで排除することが可能となった。このように、本研究により理想的なヒト正常細胞の生体外増幅法が開発された。

F. 健康危険情報

該当なし

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H. 知的財産権の出願・登録状況
該当なし

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A．研究目的
高度な歯槽骨の吸収を起こした歯周病に対する治療法の開発は、歯科医師の悲願であり、高齢化社会を迎えた日本の重要課題の一つである。骨芽細胞あるいは間葉系幹細胞はその治療材料として有用であると考えられるが、その有用性は十分に検討されていない。歯髄組織から歯髄幹細胞を単離し、それを応用できれば、患者への侵襲が少なく採取が比較的容易であるため、有効な歯周組織再生治療の材料となりえる。そこで本研究では歯髄組織から幹細胞画分を採取し、その性状を解析した。

B．研究方法
ブタ歯髄組織においてHoechest33342を強く排出する細胞、side population (SP)細胞を分離した。得られたSP細胞について、各種幹細胞マーカーの解析をRT-PCR法およびフローサイトメトリーを用いて解析した。また、各種増殖因子を用いて、骨様組織、軟骨組織、および脂肪組織形成能などを検討した。さらに、マウスの下肢虚血モデルを用いて、同幹細胞画分の血管再生能を in vivoで検証した。
（倫理面への配慮）
本研究で用いる遺伝子操作や疾患モデル動物については、所属研究機関の各専門委員会の承認を受けて行った。また、疾患モデル動物の処置については動物愛護精神にのっとり慎重に行った。剖検脳の解析にあたっては所属研究機関の倫理委員会の承認を得て行った。

C．研究結果
SP 、non SP、primary 細胞の継代培養を行い経時的に総細胞数を測定すると non SP、primary細胞は 10代目で SP細胞は19代目で平衡に達した。リアルタイムRT-PCRにてBmi1やstat3を比較するとSP細胞は他の細胞に比べて高い発現がみられた。また、同細胞は間葉系幹細胞の細胞表層マーカーであるCD13, 34, 105, 146, 150の高発現が認められた。また、SP細胞を pellet culture法で三次元培養を行うと骨様組織の形成が認められたことから、同細胞が骨芽細胞様細胞へ分化することが明かになった。さらに、CD31$^-$;CD146$^-$ SP細胞はVEGF存在下で血管内皮細胞に分化し、高い遊走能、増殖能がみられた。さらに、下肢虚血マウスに同細胞を接種したところ、有意な血管再生が認められた。

D．考察
本研究の結果から、歯髄SP細胞は間葉系幹細胞としての性質を有し、骨芽細胞様細胞への分化とともに血管再生も強く誘導することから、歯槽骨を再生するための素材として有用であるばかりでなく、虚血臓器等への再生医療にも応用可能であることが示唆された。 特に、CD31$^-$;CD146$^-$ SP細胞は口腔組織の再生に有用であることが示された。今後、効率的にSP細胞画分を分離し増幅する技術を開発することが必要である。また、用いた細胞はブタ由来であるのでヒト歯髄においても同様の細胞は採取できるのか確認する必要がある。また、ヒトへの応用に際した安全性、有効性の確認も並行して行う必要がある。

E．結論
ブタ歯髄由来SP細胞が間葉系幹細胞の性質を有し、骨形成能とともに血管再性能

も有することが明らかになった。特に、CD31⁻;CD146⁻ SP細胞が有用であることが示された。

F．健康危険情報
なし

G．研究発表
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H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし
2. 実用新案登録
なし
3.その他
なし

II. 研究成果に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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III. 研究成果の刊行物・別冊

The KK-Periome Database for Transcripts of Periodontal Ligament Development

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ABSTRACT The periodontal ligament (PDL) is a strong connective tissue that surrounds the tooth root, absorbs occlusal forces, and functions as a sense organ. PDL originated from dental follicle (DF), which possessed mesenchymal progenitors in the developing tooth germ. However, as specific marker genes for PDL and DF are currently unavailable, the molecular mechanisms of PDL development are yet to be clarified. To facilitate the identification of such genes, we have previously established a transcriptome database of the human PDL (the KK-Periome database) and screened for specific genes expressed during PDL development. Initial screening of the database revealed two marker genes for distinguishing DF and PDL. The KK-Periome database thus appears to offer a useful resource for investigating genes involved in PDL development. *J. Exp. Zool. (Mol. Dev. Evol.) 312B, 2009.* © 2009 Wiley-Liss, Inc.

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The periodontium is a tooth-supporting tissue comprising gingiva, periodontal ligament (PDL), cementum, and alveolar bone (Ten Cate, '94). More specifically, the PDL is an element that connects the cementum covering the tooth root surface and the alveolar bone of the maxilla or mandible (Ten Cate and Mills, '72; Beertsen et al., '97; McCulloch et al., 2000). The PDL has the ability to absorb mechanical stress, and has thus been compared with tendon and ligament (McCulloch et al., 2000; Yoshizawa et al., 2004). Similar to tendons and ligaments, collagen type I is predominant, although other types of collagen (types III, V, VI, and XII) and proteoglycans that regulate collagen fibril formation are also deposited in the PDL (Lukinmaa and Waltimo, '92; Liu et al., '95; Everts et al., '98; MacNeil et al., '98). PDL cells also express genes involved in tendon and ligament formation, such as scleraxis and growth and differentiation factors-5, -6, and -7 (Sena et al., 2003; Seo et al., 2004; Yokoi et al., 2007). The cellular content of PDL is predominated by PDL cells, which can form Sharpey's fibers, collagen fibers embedded into the calcified matrix (McCulloch et al., 2000; Nanci and Bosshardt, 2006). Previous findings have suggested that although cultured PDL cells fail to form mineralized nodules, a partial resemblance to osteoblasts is apparent, such as high alkaline phosphatase activity and expression of osteoblast marker genes including RUNX2, bone sialoprotein, and type I collagen (Yamashita et al., '87; Nojima et al., '90; Lekic et al., 2001; Saito et al., 2002; Murakami et al., 2003). Recently, regulatory mechanisms preventing osteoblastic differentiation have been identified in cultured PDL cells, suggesting that the ability for suppressing osteoblast function may

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act to prevent PDL from mineralizing (Yoshizawa et al., 2004; Yamada et al., 2007).

The structure of the PDL is often irreversibly damaged when chronic inflammation in the form of "periodontitis" develops, suppressing the periodontium (Bartold and Narayanan, 2006). Although various treatments are available for periodontitis, reliable regeneration of the PDL is not yet possible (Melcher et al., '86; Somerman et al., '99; Bartold et al., 2000; Shimono et al., 2003). A basic understanding of PDL development at the molecular level is required to develop regeneration therapy of PDL, as the regeneration process needs to mimic the cellular events of PDL development (Grzesik and Narayanan, 2002). Recent advances have revealed the existence of progenitor or stem cells in adult PDL (Handa et al., 2002a; Seo et al., 2004; Fujii et al., 2008). To clarify the differentiation mechanisms of these cells, it is necessary to investigate the molecular basis of PDL development.

Progenitor cells are generated by multipotent stem cells and can differentiate into one specific type of cell depending on the cellular environment during organogenesis (Potten and Loeffler, '90). In the case of PDL development, dental mesenchyme generates the dental follicle (DF), which contains PDL cell progenitors that contribute to the formation of the PDL (Cho and Garant, 2000). However, the molecular aspects of PDL development are only vaguely understood owing to the limited information available on marker genes for PDL cell progenitors and PDL cells. Conversely, thanks to recent advances in the availability of genome sequence information for both human and mouse, transcriptomes for various tissues, and bioinformatics for database construction, the identification of genes potentially involved in the development of specialized tissues has become possible (Sunkin and Hohmann, 2007). We describe herein the initial screening of potential marker genes for PDL cell progenitor and PDL cells from the KK-Periome database, which is a collection of transcripts expressed during human PDL development. The significance of extracellular matrix (ECM) components as markers for PDL development is discussed.

MOLECULAR MECHANISMS OF PDL DEVELOPMENT

The PDL originates from the DF formed during the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from cranial neural crest cells (Chai et al., 2000; Cho and Garant, 2000). DF is formed from cranial neural crest-derived dental mesenchyme on embryonic day 15 (E15) mouse embryo at the stage of tooth germ development (Fig. 1). As DF contains three kinds of progenitor cells (cementoblast, PDL cell, and osteoblast progenitors), developmental events in the DF are of considerable interest (Morotome et al., '98; Hou et al., '99; Saito et al., 2005). At the cap stage in E15 mouse embryo, dental mesenchyme undergoes differentiation into two distinct types of cells: dental papilla (DP) cells and DF cells (Chai et al., 2000; Cho and Garant, 2000). At the E17 bell

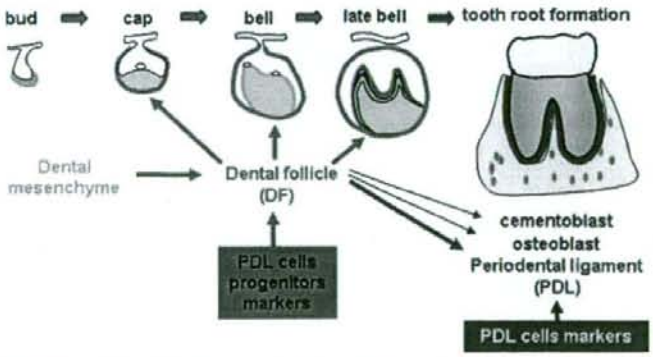

Fig. 1. Schematic of periodontal ligament development. The dental follicle (DF) is initially formed from dental mesenchyme in the cap stage of tooth germ, and differentiates to progenitors of the periodontal ligament (PDL), cementoblasts, and osteoblasts during the bell and late bell stages. After birth, differentiation of progenitors within the DF forms periodontium during the tooth root formation stage. Investigation of the molecular mechanisms underlying the PDL development requires the identification of specific marker for PDL cell progenitors and PDL cells.

stage, DP cells differentiate into cells of the odontogenic lineage, such as odontoblasts or dental pulp cells, eventually giving rise to the dentin–pulp complex in the tooth (Thesleff et al., 2001). After birth, differentiation begins during tooth root formation when cementoblast progenitors migrate to the surface of the tooth root and differentiate into cementoblasts to form cementum matrix (Bosshardt and Schroeder, '96; Saito et al., 2001). At almost the same time, PDL progenitors differentiate into PDL on cementoblasts, inserting collagen fibers known as Sharpey's fibers into the cementum matrix. Fiber insertion also takes place along the alveolar bone. Finally, both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus, which resembles tendinous tissue (Johnson, '87; Cho and Garant, '89, '96).

The fate of progenitor cells in the DF has been investigated using cultured DF cells. Because the experimental model for in vitro differentiation of PDL is not available, implantation analysis using immunodeficiency mice has been used for studying the potential of PDL formation. For instance, cultured DF cells do not form mineralized nodules in vitro even after treatment with mineralization-inducing medium, but are able to form PDL-like tissue and cementum-like structures after implantation into immunodeficient mice (Handa et al., 2002b; Yokoi et al., 2007). In addition, progenitor cell lines for cementoblasts and PDL have been established from immortalized DF cells using single cell cloning methods (Morsczeck et al., 2005; Saito et al., 2005; Luan et al., 2006). These findings support the notion that PDL cell progenitors are present in DF. Investigation of the differentiation of PDL cell progenitors into PDL has thus enabled clarification of transcription factors, signaling molecules, and specific niches involved in PDL differentiation. However, elucidation of the precise differentiation mechanisms for PDL cell progenitors in DF has been hampered by the paucity of specific marker genes for these cells (Pitaru et al., '94; Diekwisch, 2002; Nanci and Bosshardt, 2006). Identification of specific markers that can distinguish PDL cell progenitors and PDL cells is thus important (Fig. 1).

FUNCTIONAL MOLECULES INVOLVED IN PDL DEVELOPMENT

Although no specific marker for PDL is available, several reports have shown that PDL development seems to be dependent on the ECM, which regulates collagen fibril formation and is mainly deposited in PDL (McCulloch, 2000). Type I and III collagens are the major components of collagen bundles and Sharpey's fibers in PDL, and fibril assembly is responsible for the structural stabilization and mechanical characteristics of the PDL (Ten Cate, '94; MacNeil et al., '98). Interactions between fibrillar collagen and small leucine-rich proteoglycans (SLRPs) such as lumican and decorin regulate flexible connective tissues such as tendons (Danielson et al., '97; Ezura et al., 2000; Matheson et al., 2005). Lumican- and decorin-deficient mice show abnormal collagen fibrils in PDL, indicating that these SLRPs regulate the organization of collagen fibrils during PDL formation (Matheson et al., 2005). Conversely, Yamada et al. (2001, 2007) found that PDL-associated protein (PLAP)-1/asporin, which belongs to a novel SLRP family, is specifically expressed not only in DF but also in adult PDL. PLAP-1/asporin directly interacts with bone morphogenetic protein-2 to inhibit the mineralization of the PDL. This finding strongly suggests that PDL synthesizes PLAP-1/asporin as a negative regulator of mineralization to prevent ankylosis. Noncollagenous ECM is also involved in the formation of PDL. Periostin is a secreted adhesion protein that displays homology with fasciclin I, an insect growth cone guidance protein (Horiuchi et al., '99). During tooth germ development, periostin is initially expressed in the DF, and then becomes restricted to postnatal PDL during tooth root formation (Kruzynska-Frejtag et al., 2004). Periostin-deficient mice show disorganization of the PDL and alveolar bone resorption, suggesting a critical role of this protein in the maintenance of PDL and onset of periodontal disease (Rios et al., 2005; Kii et al., 2006).

As PDL is morphologically similar to tendon and ligament, the suggestion has been made that tendon/ligament phenotype-related genes that are specifically expressed during the formation of tendons and ligaments are involved in the differentiation of PDL cell progenitors (Table 1) (Oliver et al., '95; Wolfman et al., '97; Brent et al., 2003; Salingcarnboriboon et al., 2003). Based on these observations, the ECM predominantly deposited in PDL and tendon/ligament-related genes that are expressed in PDL may contribute to the formation of PDL. However, it is difficult to investigate whether these factors are involved in PDL development owing to the lack of specific markers for PDL cell progenitors and PDL cells.

TABLE 1. List of tendon- and ligament-related genes

Functional categorization	Gene name	References
Transcription factor	Scleraxis	Brent et al. (2003)
	Six1 Six2	Oliver et al. ('95)
Plasma membrane protein	EphA4	Patel et al. ('96)
Signaling molecules	GDF5	Sodersten et al. (2005) and Wolfman et al. ('97)
	GDF6 GDF7	

Genes expressed in tendon and ligament are listed according to functional categorization. GDF, growth and differentiation factor.

THE KK-PERIOME DATABASE AND SCREENING OF SPECIFIC MARKER FOR PDL CELL PROGENITORS AND PDL CELLS

To identify specific markers for PDL cell progenitors and PDL cells, we established the KK-Periome database as a collection of 617 clusters of expressed sequence tags (ESTs) highly expressed in human PDL (Nishida et al., 2007). These EST clusters were derived from short single-pass sequence reads of 11,520 randomly selected clones from the human PDL cDNA library and were considered invaluable for identifying particular genes and unique gene expression patterns during PDL development. For the identification of specific markers for PDL cell progenitors and PDL cells from the KK-Periome database, screening of candidate genes was performed using two different criteria of functional classification and expression pattern (Fig. 2). As ECM components are considered to be involved in the determination of cell specificity (Engler et al., 2006; Scadden, 2006), we hypothesized that the DF and the PDL are each composed of specific ECM proteins, and that these unique matrices could serve as specific markers for PDL cell progenitors or PDL cells. As a result of functional classification, we obtained 38 ECM clusters from KK-Periome database, which include type I collagen α 2, Secreted proteins acidic, cystein-rich (SPARC)/osteonectin, collagen type III, periostin, lumican, type I collagen α I chain, osteopontin, decorin, fibronectin, and PLAP-1/asporin as the ten most highly expressed transcripts. Most of the ECM proteins present here for the characterization of the PDL are ubiquitous molecules although the PDL has a unique structure and function (Yamada et al., 2001). Therefore, we next screened ECM proteins that had not previously been reported in PDL according to a PubMed search, and 13 ECM clusters were obtained as candidates, including the genes for collagen type XI α 1, SPARC-like 1, tenascin-N, collagen type XV α 1, hyaluronan and proteoglycan link protein 1, vitrin, type XVI collagen α 1, SPARC-related modular calcium binding 2, granulin, fibulin 5, F-spondin, nidogen 1, and leprecan 1. We next investigated temporal and spatial expression patterns of these 13 candidate genes using in situ hybridization analysis with mouse DF and PDL tissues. As a result, two genes

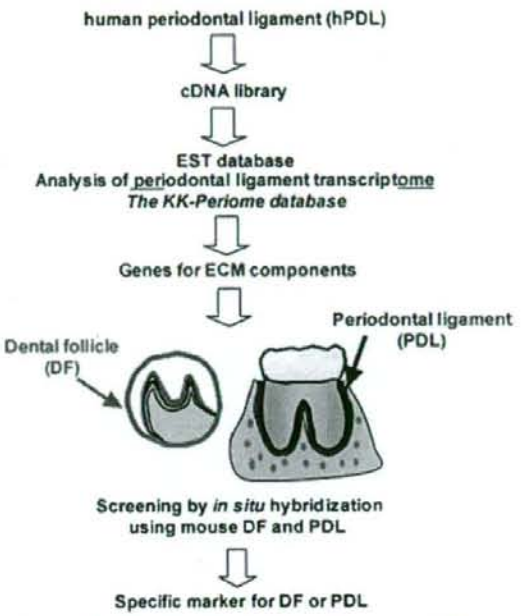

Fig. 2. Strategy for creating a transcriptome database for PDL (KK-Periome database) and a screening marker for PDL cell progenitors and PDL cells. The KK-Periome database was established following the expressed sequence tag (EST) sequence analysis of the human PDL cDNA library. Genes for ECM components including collagen type XI α 1, SPARC-like 1, tenascin-N, collagen type XV α 1, hyaluronan and proteoglycan link protein 1, vitrin, type XVI collagen α 1, SPARC-related modular calcium binding 2, granulin, fibulin 5, F-spondin, nidogen 1, and leprecan 1 were isolated and specific marker for PDL cell progenitors and PDL were screened by in situ hybridization analysis of mouse DF and PDL. PDL, periodontal ligament; ECM, extracellular matrix; DF, dental follicle.

(F-spondin and tenascin-N) were identified as candidate markers for the DF and the PDL, respectively. Interestingly, F-spondin was initially expressed in the DF at the cap stage and became progressively more evident in the DF at the bell stage. In addition, no expression of F-spondin was observed in other types of cells in the tooth germ, including DP, dental epithelium, odontoblasts, or ameloblasts. However, expression of F-spondin in DF was significantly down-regulated after birth and completely absent in the PDL of adult mice. Unlike F-spondin, expression of tenascin-N was not detected in DF cells of the developing tooth germ in the embryo, but was strongly induced in adult PDL. Although PDL contained several types of cells including PDL cells, epithelial cells of Malassez, endothelial cells, osteoblast progenitors, and cementoblast progenitors (McCulloch et al., 2000), the expression of tenascin-N is restricted to adult PDL cells. Based on these observations, F-spondin and tenascin-N may serve as specific markers for DF or PDL, respectively (Fig. 3).

F-SPONDIN AND TENASCIN-N AS MARKERS FOR PDL CELL PROGENITORS AND PDL CELLS

F-spondin is a component of the ECM, which is known to be present in the embryonic floor plate of vertebrates (Klar et al., '92; Tzarfati-Majar et al., 2001) and the caudal somite of birds (Debby-Brafman et al., '99), apparently playing a dual role in the patterning of the nervous system. F-spondin promotes the adhesion and the outgrowth of axons, but inhibits the adhesion of neural crest cells. As F-spondin-knockout mice or transgenic mice expressing F-spondin specifically in PDL are not available, the precise function of F-spondin during PDL development is unclear. However, transient expression of F-spondin in DF strongly suggests the involvement of PDL development, rather than the maintenance of adult PDL function such as withstanding force of mastication. F-spondin was highly expressed in a human cementoblast cell line, and overexpression induced the up-regulation of cementoblast/osteoblast mar-

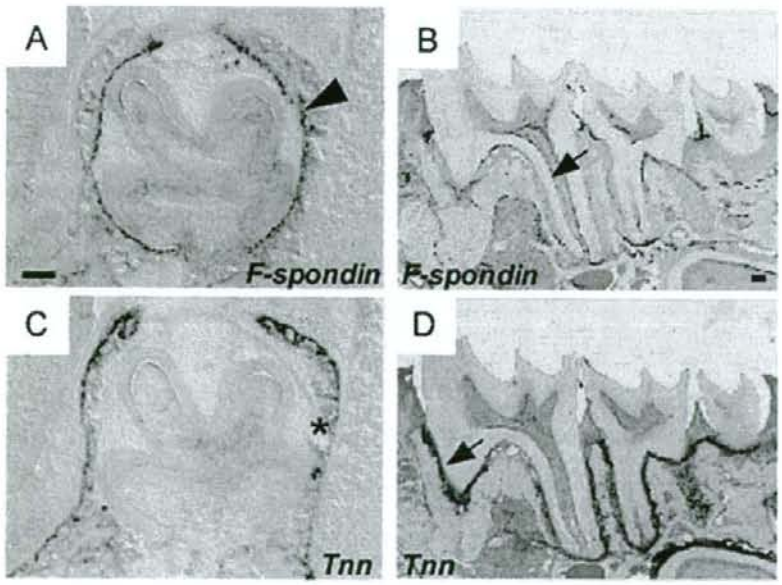

Fig. 3. Expression of F-spondin and tenascin-N during PDL development. In situ hybridization analyses of *F-spondin* (**A** and **B**) and *tenascin-N* (**C** and **D**) in the late bell stage of tooth germ (A and C) and adult molar (B and D) are shown. Note that *F-spondin* is intensely expressed in DF (A, arrowhead), whereas expression is significantly down-regulated in PDL (B, arrow). In contrast, *tenascin-N* (*Tnn*) is strongly expressed in the PDL (D, arrow), but not expressed in the DF (C, asterisk). Bar, 50 μm. Reprinted from Nishida et al. 2007. Transcriptome database KK-Periome for periodontal ligament development: expression profiles of the extracellular matrix genes. Gene 404:70–79 with permission from Elsevier. PDL, periodontal ligament; DF, dental follicle.

ker gene such as bone sialoprotein and osteocalcin (Kitagawa et al., 2006). Moreover, F-spondin protein was detected in the cementum matrix. Contrasting with these results, we found high levels of F-spondin mRNA in the DF and dramatic decreases in the adult PDL suggesting that F-spondin may involve in the formation of DF.

Tenascin-N is a novel member of the tenascin family of proteins, which are expressed in the brain, kidneys, and spleen of adult animals. Unlike other tenascins, tenascin-N is highly expressed in neurons of the central nervous system (Neidhardt et al., 2003). Interestingly, the expression of tenascin-N was strongly up-regulated in the adult PDL, whereas no expression was observed in the DF. Periostin has been used as a marker for distinguishing PDL from adjacent connective tissues such as bone and gingiva in adult tissue (Horiuchi et al., '99; Kruzynska-Frejtag et al., 2004). The expression of periostin was detected in both adult PDL and DF in the developing tooth germ. In contrast to periostin, tenascin-N was specifically expressed in adult PDL (Nishida et al., 2007). In addition, the expression patterning of PLAP-1/asporin resembled that of periostin during PDL development (Yamada et al., 2007). Tenascin-N was thus considered to serve as a distinct marker of differentiated PDL cells. Furthermore, tenascin-N was detected in the costal perichondrocytes that eventually form the ligament tissue suggesting that the expression may be associated with the formation of ligamentous tissues (Nishida et al., 2007).

CONCLUSION

PDL development requires PDL cell progenitors present in the DF during tooth germ development (Cho and Garant, 2000). Although stem/progenitor cells capable of forming PDL have been identified (Seo et al., 2004; Yokoi et al., 2007; Fujii et al., 2008), the precise differentiation mechanisms of these cells remain unclear owing to the unavailability of markers for PDL cell progenitors and PDL cells. A combination of the transcriptome database for the human PDL (KK-Periome database) and in situ hybridization analysis helped to identify F-spondin and tenascin-N as specific markers for PDL cell progenitor or PDL cells, respectively (Fig. 4). Although functional roles of F-spondin and tenascin-N are unclear, development of the PDL is apparently associated with changes in the expression of these proteins. To investigate whether these molecules are involved in PDL formation, development of functional assay system either in vitro or using transgenic mice is necessary. However, further screening of the KK-Periome database may provide more novel marker genes that are useful for analyzing the molecular mechanisms of PDL development and identification of eventual therapeutic targets for the treatment of periodontal disease.

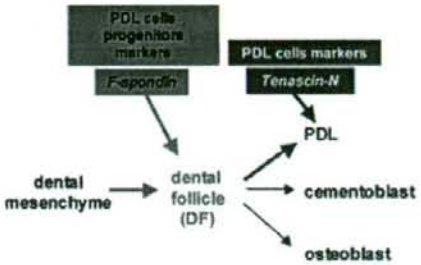

Fig. 4. F-spondin and tenascin-N as markers for PDL cell progenitors or PDL cells. F-spondin is specifically expressed in DF, whereas the expression is significantly down-regulated in PDL. In contrast, expression of tenascin-N is strongly induced in the PDL. We thus proposed F-spondin and tenascin-N as markers for PDL cell progenitors or PDL cells. PDL, periodontal ligament; DF, dental follicle.

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